

## Original article

# *Annona senegalensis* extract demonstrates anticancer properties in *N*-diethylnitrosamine-induced hepatocellular carcinoma in male Wistar rats

Omolara F. Yakubu<sup>a,\*</sup>, Damilohun S. Metibemu<sup>b</sup>, Isaacson B. Adelani<sup>a</sup>, Gbolahan O. Adesina<sup>a</sup>, Chidera B. Edokwe<sup>a</sup>, Oyinkansola E. Oseha<sup>a</sup>, Abiodun H. Adebayo<sup>a,\*</sup>

<sup>a</sup> Department of Biochemistry, College of Science and Technology, Covenant University, Canaan Land, Ota PMB 1023, Ogun State, Nigeria

<sup>b</sup> Department of Biochemistry, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria

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## ABSTRACT

**Background:** Hepatocellular carcinoma (HCC) is a common and leading cancer around the globe. This study investigated the anticancer properties of extract of *Annona senegalensis* in *N*-diethylnitrosamine (DEN) - induced hepatocellular carcinoma in male Wistar rats.

**Methods:** Rats were simultaneously induced with a combination of 100 mg/kg b.wt of DEN and 0.5 mL/kg of carbon tetrachloride (CCl<sub>4</sub>) intraperitoneally once a week for three weeks in a row. Thereafter, animals were treated with 100 mg/kg and 200 mg/kg b.wt of *A. senegalensis* extract daily for 21 days. Analysis using gas chromatography-mass spectrometry (GC-MS) was carried out to discover the phytoconstituents contained in the n-hexane extract of *A. senegalensis*. The levels of liver function parameters and antioxidant enzyme activities were determined via spectrophotometric analysis. Reverse transcriptase-polymerase chain reaction technique was used to assess the gene expression patterns of BCL-2, P53, P21, IL-6, FNTA, VEGF, HIF, AFP, XIAP, and EGFR mRNAs.

**Results:** Treatment of DEN-induced hepatocellular carcinoma Wistar rats with the extract caused significant ( $p < 0.05$ ) decrease in the activities of ALT and AST. It also resulted in a reduction of the concentration of MDA and a significant increase ( $p < 0.05$ ) in SOD and GSH activities. IL-6, BCL-2, VEGF, EGFR, XIAP, FNTA, and P21 mRNAs expressions were significantly ( $p < 0.05$ ) downregulated after treatment. Histopathological analysis revealed that the extract improved the liver architecture.

**Conclusion:** *A. senegalensis* n-hexane extract demonstrates its anticancer properties by improving the liver architecture, increasing the antioxidant defense systems, downregulating the pro-inflammatory, anti-apoptotic, angiogenic, alpha-fetoprotein and farnesyl transferase mRNAs expression and hitherto up-regulate the expression of tumor suppressor (P21 and P53) mRNAs.

## 1. Introduction

Nitrosamines are formed by combining nitrates or nitrites with amines. Diethylnitrosamine (DEN) is a type of nitrosamines, which is a known hepatocarcinogen forming DNA adducts in the liver [1]. Induction of oxidative stress by DEN further contributes to the pathogenesis of hepatocellular carcinoma [2,3]. DEN and other known hepatocarcinogens induce hepatic injury by enhancing the proliferation of cells, which is accompanied by hepatocellular necrosis [4,5]. They target the liver where they are bio-transformed via the cytochrome p450-dependent mechanism, CYP2E1, explicitly resulting in DNA-adducts [6,7]. Hepatic carcinogenesis is ranked the fifth most prevalent

cancer and usually influenced by agents such as alcohol, phenobarbital, 2-acetylaminofluorene, and DEN [8]. Animal models are viewed as a critical tool for studying hepatic carcinogenesis and are often used for cancer research. Studies have reported early induction of oxidative stress, inflammation, and proliferation by DEN in rat models [9,10].

Medicinal plants and herbs contain phytochemicals and natural sources of antioxidants. The significance of these active metabolites has prompted important scientific interest in their biological activities. *Annona senegalensis* (AS) is one such plant containing an abundance of phytochemicals; it contains alkaloids, flavonoids, glycosides, tannins, saponins, steroids, and anthocyanins [11–13]. AS is often referred to as wild soursop; the plant is a 2–5 m tall shrub having blue to greenish,

\* Corresponding authors.

E-mail addresses: [omolara.yakubu@covenantuniversity.edu.ng](mailto:omolara.yakubu@covenantuniversity.edu.ng) (O.F. Yakubu), [abiodun.adebayo@covenantuniversity.edu.ng](mailto:abiodun.adebayo@covenantuniversity.edu.ng) (A.H. Adebayo).

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oblong, alternate, and simple leaves. It is prominent among the Northern region of Nigeria and commonly called in Hausa language, Gwándàn dààjìlì [14,15]. Almost all the plant parts have been reported useful for medicinal applications. The leaves are used for yellow fever, tuberculosis, and smallpox treatment while the stem bark is employed for treating hernia and snakebite [16,17]. The root is employed to ease swallowing, impotence, and an antidote for poison; root bark helps in the treatment of infectious diseases while the juice from the plant dries up chickenpox on the skin [18–20]. Several studies have documented its biological activities such anticonvulsant [21–24], antimicrobial [25], antiplasmodial [26], anti venomous [27,28], spermatogenic [29], anti-inflammatory, analgesic [30,31] and antihelminthic activities [32, 33]. This study aimed to ascertain the anticancer effect of *Annona senegalensis* leaf extract on hepatocellular carcinoma induced by diethylnitrosamine in Wistar rats.

## 2. Materials and methods

### 2.1. Collection and identification of plants

*A. senegalensis* leaf samples were collected around Ile-Ife in Osun State, Nigeria. A botanist, Dr. J.O. Popoola from Biological Science Department, Covenant University, Nigeria, identified the leaf. Samples were deposited in the Herbarium at the Forest Research Institute of Nigeria (FRIN) with voucher no 112597.

#### 2.1.1. Processing of plant samples

The leaves were processed using methods by Younoussa et al. [34]. Fresh leaves of *A. senegalensis* was dried with ambient air at 25 °C for eight weeks, after which an electric blender was used to pulverize it. The powdered leaves (4.5 kg) were further soaked for 72 h in N-hexane, filtered using a filter paper (Whatman No. 2) and concentrated using a rotary evaporator at 40 °C to give a final weight of 161.8 g of the extract and percentage yield of 3.6.

### 2.2. Gas chromatography–mass spectrometry analysis

This was carried out with the aid of Agilent Technologies (6890 Series) GC connected to (5973 Series) Selective Mass Detector. The carrier gas (helium) with the oven temperature set at 50 °C (for 2 min) and raised steadily at 4 °C/minute; after which 2.0 µL of the diluted sample was introduced at a ratio of 1–50 (1:50). The GC model series 6890 was linked to the NIST/NBS 20 database for comparing the compounds' mass spectra to authentic samples.

### 2.3. Chemicals

N-Diethylnitrosamine (DEN) and carbon tetrachloride (CCl<sub>4</sub>) were purchased from AK Scientific USA and local vendors, respectively. The test kits were procured through local vendors from Randox Laboratories. All other chemicals used were of analytical grades.

### 2.4. Animals

Thirty-six healthy six-week-old male rats (150 g) were obtained from the Animal Holding unit of Lagos State University Teaching Hospital, Nigeria. They were housed in clean plastic cages with adequate ventilation conditions (12/12 h light/dark cycle) and given food and water ad libitum, acclimatized for two weeks. The rats were maintained following the National Institute of Health guidelines on handling laboratory animals [35]. Ethical approval was obtained from the Research Ethical Committee of Covenant University, Ota, Nigeria, and was assigned an approval no NHREC/25/10/2018.

### 2.5. Study design

The rats (36) were grouped into six groups. Three groups (B to D) were induced intraperitoneally (IP) with 100 mg/kg of DEN and 0.5 mL/kg b.wt of carbon tetrachloride (CCl<sub>4</sub>) weekly for the first three weeks while groups A, E, and F took corn oil (the vehicle). Treatment of groups C and D started by the 4th week with 100 and 200 mg/kg of the extract respectively for three weeks; 100 and 200 mg/kg body weight of extract were administered to groups E and F respectively while group A received the vehicle all through the experiment [36].

A (Control): Standard diet and corn oil

B (Negative control): Intraperitoneal administration with 100 mg/kg of DEN and 0.5 mL/kg b.wt of carbon tetrachloride (Toxicant)

C: Toxicant +100 mg/kg b.wt of *A. senegalensis*

D: Toxicant +200 mg/kg b.wt of *A. senegalensis*

E: 100 mg/kg b.wt *A. senegalensis* only

F: 200 mg/kg b.wt *A. senegalensis* only

At the end of the treatment, the rats were fasted overnight and sacrificed the next morning via cervical dislocation. Samples were collected for biochemical assays, and the liver was excised, weighed, and a portion was fixed in 10 % formalin while the remaining were frozen at –80 °C until needed for further analysis.

### 2.6. Biochemical assays

Biochemical parameters assayed using Randox commercial test kits include the following; alanine and aspartate aminotransferases (ALT and AST) [37,38], alkaline phosphatase (ALP) [39], and lactate dehydrogenase (LDH) [40] were determined using Randox test kits and following the manufacturer's instruction. Thiobarbituric acid reactive substances (TBARS) was measured to ascertain the microsomal lipid peroxidation levels [41]. Other antioxidant parameters include: reduced glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were analyzed following standard procedures [42–44].

### 2.7. RNA isolation and reverse transcription polymerase chain reaction

TRIzol reagent (Gibco) was used for RNA isolation from the liver tissues; RNA-free DNase (Roche, Switzerland) was added to the isolated RNA and left at 37 °C for fifteen minutes while the RNeasy kit (Qiagen, Germany) was used for RNA purification. 40 µg of the RNA was allowed to incubate with the reverse transcriptase (GE Healthcare, UK) for an hour together with hexanucleotides for the synthesis of cDNA, adhering to the manufacturer's instructions. This was followed by PCR amplification using specific primers (Table 1) for 50 cycles using a thermal cycler (BioRad, USA). The amplicon bands were analyzed on 1% agarose

**Table 1**

List of target genes and specific primer sequences

TARGET GENE	FORWARD 5'-3'	REVERSE 5'-3'
FNTA	GTGATCGCGCTGTGTGGAG	CGCACTCTCATTGTGTGGC
IL 6	GACTTCCAGCCAGTTGCCTT	GCAGTGGCTGTCAACAACAT
P21	GAGAACTGGGGAGGGCTTTC	TCCTGAGCCTGTTTCGTGTC
VEGF	CGGGCCTCTGAAACCATGAA	GCCTTCTGCTCCCTTCTGT
HIF	GCAACTGCCACCACTGATGA	GCTGTCCGACTGTGAGTACC
AFP	CACCATCGAGCTCGGCTATT	TCCCAAAACTCGCTTGGGT
P53	CCCTTGAAGACTGGATAACTGT	TCTCTGACTCAGAGGGAGC
XIAP	TGCATAATGAGGACTGGGGG	GCCCGATCAGGAAAAACAC
EGFR	GATTAATCCCGAGAGGCCAGA	TGAGCCTGTTACTTGTGCCT
BCL-2	GGTGAAGTGGGGAGGATTG	AGAGCGATGTTGTCCACCAG
β-actin	CCGCAGACTTGGAGAAAGT	GTTTCGATGGCCTTCATCGC

\*FNTA: Farnesyltransferase, IL-6: Interleukin-6, VEGF: Vascular endothelial growth factor, HIF: Hypoxia-inducible factors, AFP: Alpha-fetoprotein, XIAP: X-linked inhibitor of apoptosis protein, EGFR: Epidermal growth factor receptor, BCL-2: B-cell lymphoma-2.

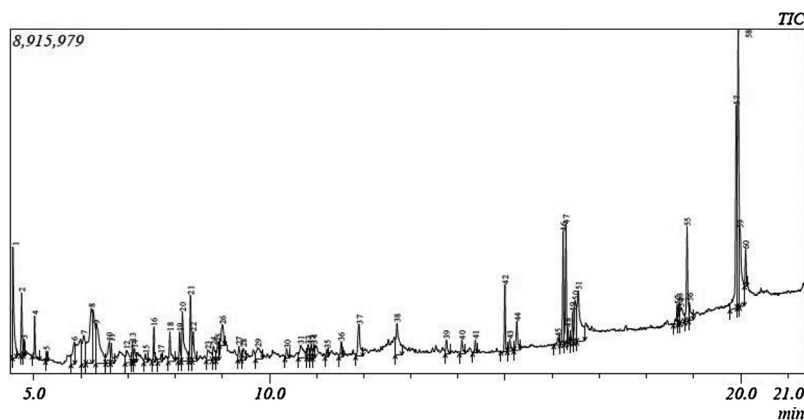


Fig. 1. Chromatogram of *A. senegalensis* extract.

and stained with ethidium bromide [45].

## 2.8. Histopathology

Liver tissues fixed in formalin (10 %) were sliced into to 2.1 mm thick; dehydrated with ethanol, handled with paraffin wax, and then cast into blocks; sections of the tissue were diced into 5  $\mu$ m and left to dry on a slide. Haematoxylin-eosin stain was used to stain the blocks and then analyzed using a light microscope [46,47].

## 2.9. Statistical analysis

GraphPad (6.0) was used for the statistical analysis, while group comparisons were done using one-way ANOVA. All values were reported as mean  $\pm$  SEM (standard error of the mean) using a 95 % confidence interval.

## 3. Results

### 3.1. GCMS analysis

The analysis revealed 60 bioactive compounds (Fig. 1 and Table S1). The compound identified having the lowest retention time of 4.56 min was Dimethyl Sulfoxide, while the Oleoyl chloride had a retention time of 19.98 min. It is believed that different phytoconstituents are responsible for the biological activities of the crude extract [48]. Fig. 1 and Table S1 show the chromatogram and the phytoconstituents of *A. senegalensis* n-hexane extract, respectively.

### 3.2. Effects of *A. senegalensis* extract on *N*-diethylnitrosamine-induced hepatocellular carcinoma in male wistar rats on selected liver enzymes

The impact of *A. senegalensis* on serum ALT, AST, ALP, and liver homogenate LDH activities is presented in Fig. 2. Significant ( $p < 0.05$ ) increase in the activities of ALT, AST, ALP, and LDH in group 2 (negative

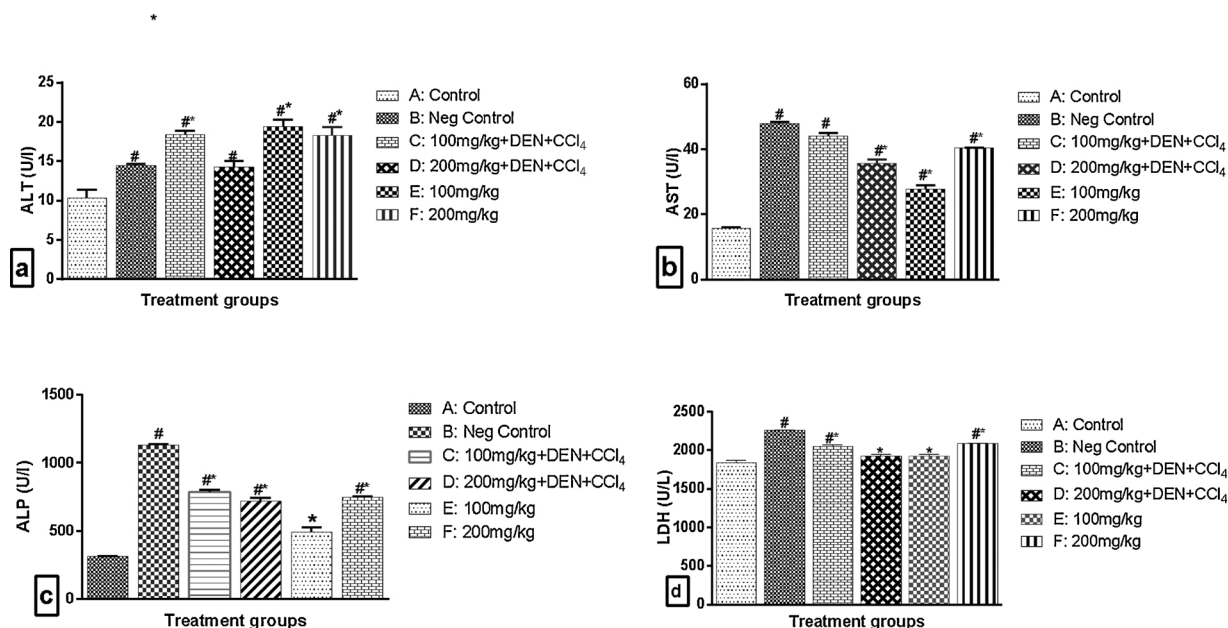
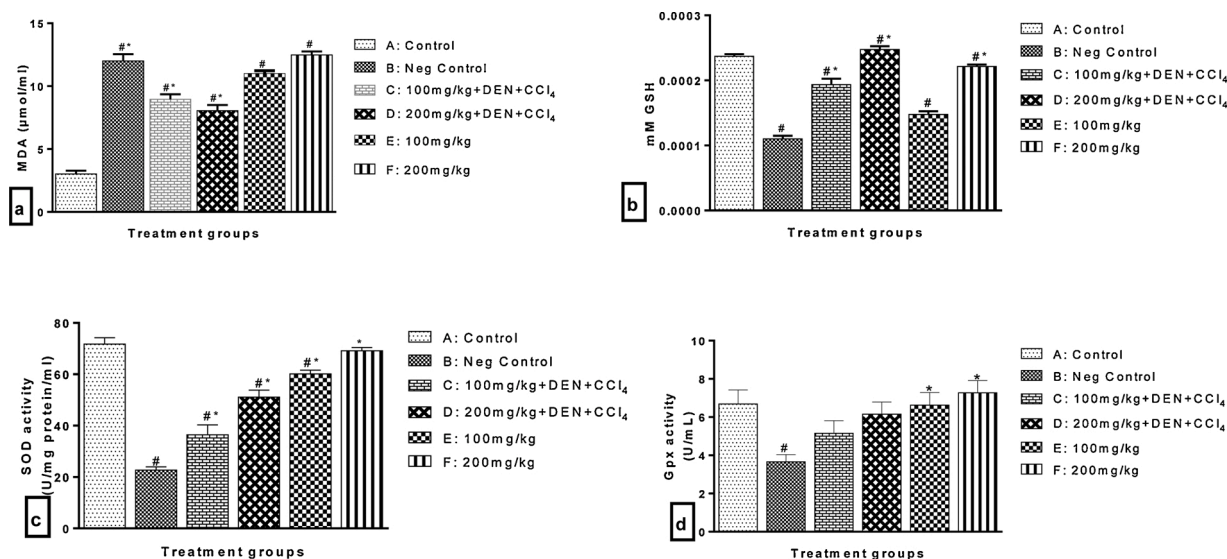
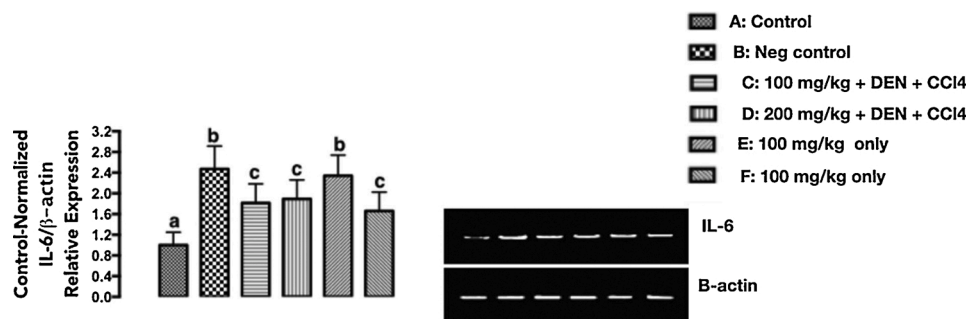


Fig. 2. Effect of *A. senegalensis* extract on liver function parameters in albino Wistar rats. Values are reported as mean  $\pm$  SEM of 6 replicates; \* $p < 0.05$  is considered significant as compared to negative control; # $p < 0.05$  is considered significant as compared to normal control. (a) ALT: Alanine aminotransferase; (b) AST: Aspartate aminotransferase; (c) ALP: Alkaline phosphatase; (d) LDH: Lactate dehydrogenase.



**Fig. 3.** Effect of *A. senegalensis* extract on antioxidant parameters in albino Wistar rats. (a) MDA: malondialdehyde; (b) GSH: reduced glutathione; (c) SOD: superoxide dismutase; (d) GPx: glutathione peroxidase. Values were reported as mean  $\pm$  SEM of 6 replicates. \* $p < 0.05$  is considered significant as compared to negative control; # $p < 0.05$  is considered significant as compared to normal control.



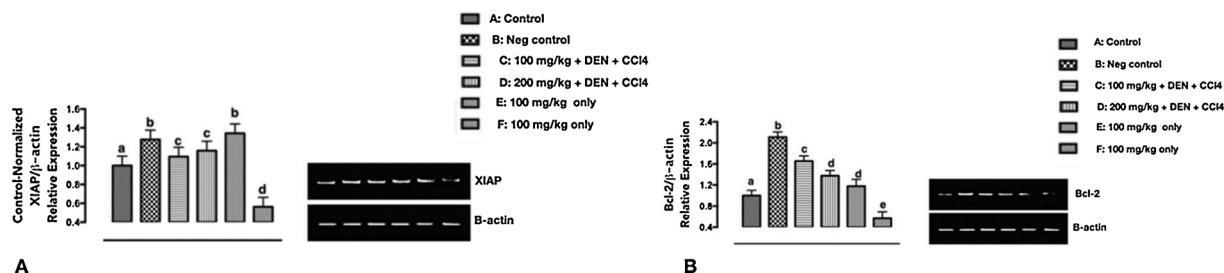
**Fig. 4.** Relative expression of IL-6 mRNA in the liver of N-Diethylnitrosamine induced-hepatocellular carcinoma rats following treatment with *A. senegalensis* extract. Bars with different alphabet depict significant differences. Bars with the same alphabet show no significant difference.  $P < 0.05$  is statistically significant.

control) was observed while upon treatment with the extract (*A. senegalensis*), the enzymes' activities were observed to be significantly reduced. Group D (Induced group treated with 200 mg/kg of extract) was found to be more effective in reducing the activities of the enzymes.

### 3.3. Effect of *A. senegalensis* extract on N-diethylnitrosamine-induced hepatocellular carcinoma in male Wistar Rats on hepatic antioxidant status

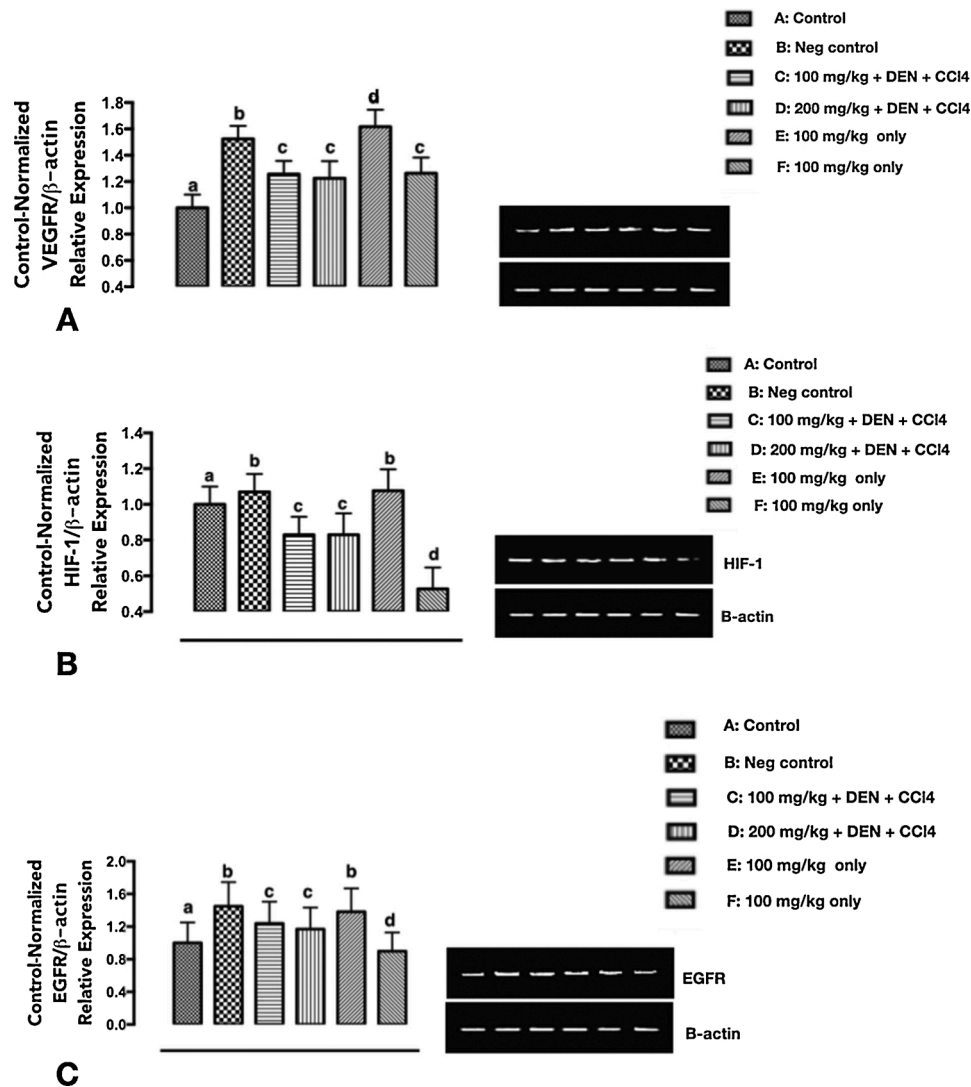
Induction of the animals with DEN without treatment (negative

control) resulted in significant ( $p < 0.05$ ) increase in malondialdehyde (MDA) concentration as compared to the control group (group A). *A. senegalensis* lowered the elevated MDA concentration in a dose-dependent manner as seen in Fig. 3. GSH level significantly ( $p < 0.05$ ) reduced in the DEN-induced untreated group (negative control) in comparison to the normal control group. Subsequent treatment with the extract produced a notable rise in the GSH level. SOD and GPx as indicators of antioxidant status were also measured in the liver tissue homogenate. Significant depletion ( $p < 0.05$ ) in their activities was observed in the untreated group as against the normal control (A) while both activities were significantly ( $p < 0.05$ ) elevated in both treated



**Fig. 5.** Relative expressions of (A) BCL-2 and (B) XIAP mRNAs in the liver of N-Diethylnitrosamine induced-hepatocellular carcinoma rats following treatment with *A. senegalensis* extract. Bars with different alphabet depict significant differences. Bars with the same alphabet show no significant difference.  $p < 0.05$  is considered to be statistically significant.





**Fig. 6.** Relative expressions of (A) EGFR, (B) HIF-1, and (C) VEGFR mRNAs in the liver of N-Diethylnitrosamine induced-hepatocellular carcinoma rats following treatment with *A. senegalensis* extract. Bars with different alphabet depict significant differences. Bars with the same alphabet show no significant difference.  $P < 0.05$  is statistically significant.

groups (C and D) as compared with the untreated group (B).

#### 3.4. mRNAs Profiling of the effects of *A. senegalensis* extract in N-diethylnitrosamine induced-hepatocellular carcinoma in Wistar rats

##### 3.4.1. Expression of inflammatory mRNA, Interleukin-6 (IL-6)

As revealed in Fig. 4, IL-6 mRNA was significantly up-regulated in the negative control (B) when compared with group A (normal control). IL-6 expression was significantly reduced by treatment with the different doses used (groups C and D). There is no significant expression of IL-6 mRNA in group E when compared with group A. However, the expression of IL-6 mRNA was significantly downregulated in group F when compared with A.

##### 3.4.2. Expression of anti-apoptotic mRNAs, X-linked inhibitor of apoptosis protein (XIAP), and B-cell lymphoma 2 (BCL-2)

In Fig. 5a, the expression of XIAP mRNA was significantly down-regulated by the treatments, groups C and D, when compared with group B. XIAP gene expression, was downregulated in group F when compared with A and B. Also, in Fig. 5b BCL-2 gene expression was significantly up-regulated in group B when compared to A. However, it was significantly down-regulated by the treatments in groups C and D on

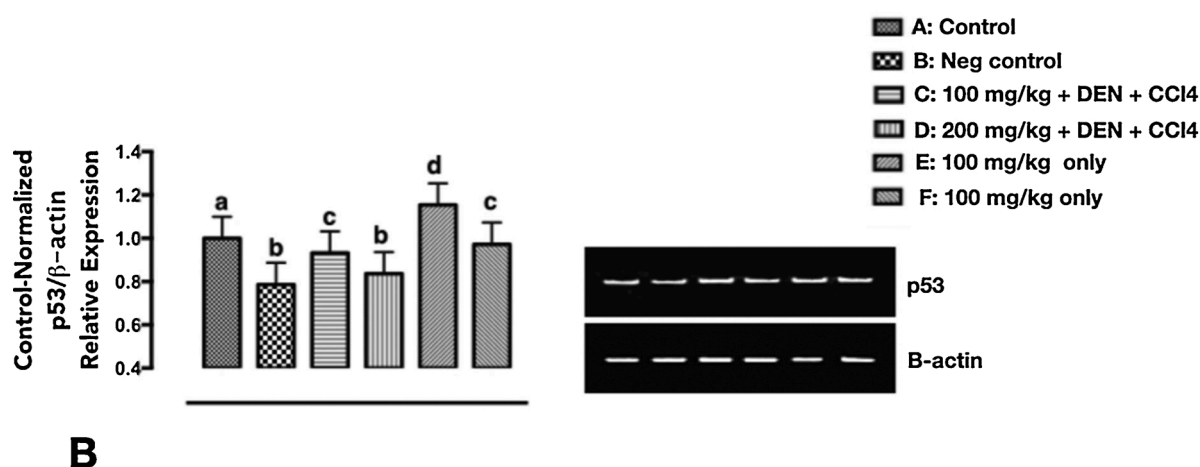
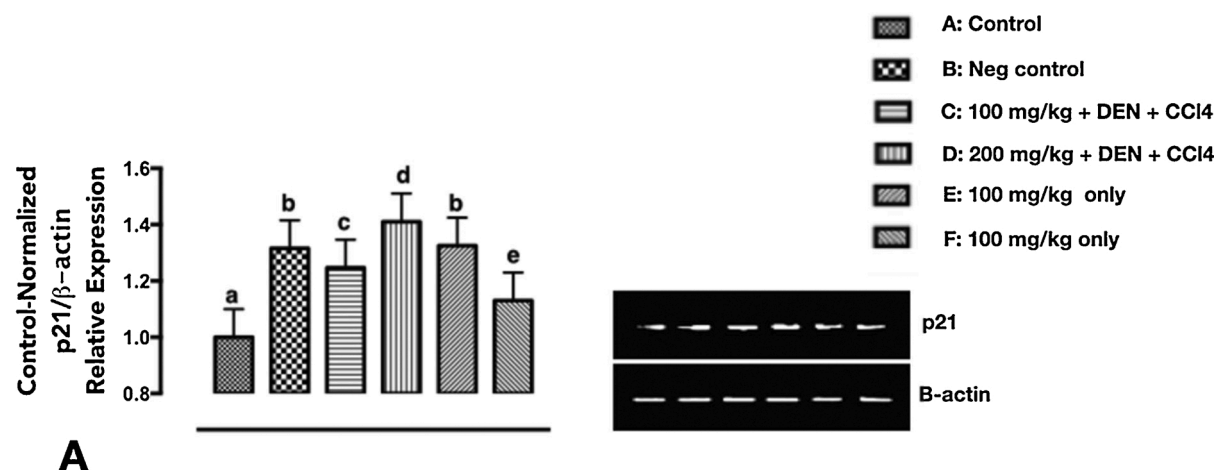
comparing with group B. There was a significant downregulation in the expression of BCL-2 gene in group F as compared with both groups A and C.

##### 3.4.3. Expression of angiogenic mRNAs, Epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and Hypoxia-Inducible Factor (HIF)-1

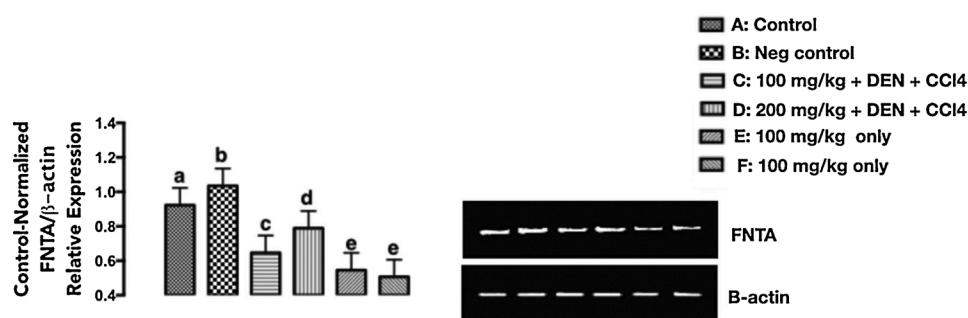
In Fig. 6, expressions of EGFR, HIF-1, and VEGFR mRNAs were up-regulated significantly ( $p < 0.05$ ) in group B when compared with group A. On the other hand, the expressions of EGFR, HIF-1, and VEGFR mRNAs were significantly downregulated by the treatments (C and D) as compared with the untreated group (B).

##### 3.4.4. Expression of cell-cycle mRNAs, p21, and p53

The p21 mRNA was significantly up-regulated in group B as compared with group A (Fig. 7a). Significant downregulation of the p21 mRNA in group C was observed when compared with group B. However, 200 mg/kg b.wt of the extract (group D) up-regulated the expression of p21 mRNA when compared with the negative control (B). A dose-dependent downregulation of p21 mRNA in groups E and F was observed when compared to group B. Fig. 7b shows the expression of p53 mRNA was significantly downregulated in the negative control



**Fig. 7.** Relative expressions of p21 and p53 mRNAs in the liver of N-Diethylnitrosamine induced-hepatocellular carcinoma rats following treatment with *A. senegalensis* extract. Bars with different alphabet depict significant differences. Bars with the same alphabet show no significant difference.  $P < 0.05$  is considered to be statistically significant.



**Fig. 8.** Relative expressions of FNTA mRNA in the liver of N-Diethylnitrosamine induced-hepatocellular carcinoma rats following treatment with *A. senegalensis* extract. Bars with different alphabet depict significant differences. Bars with the same alphabet show no significant difference.  $p < 0.05$  is significant.

when compared with the control group. p53 mRNA expression was however, significantly up-regulated in group C when compared with group B. No significant difference in the expression of p53 mRNA in group D was observed upon comparing with group B, while its expression was significantly up-regulated in group E.

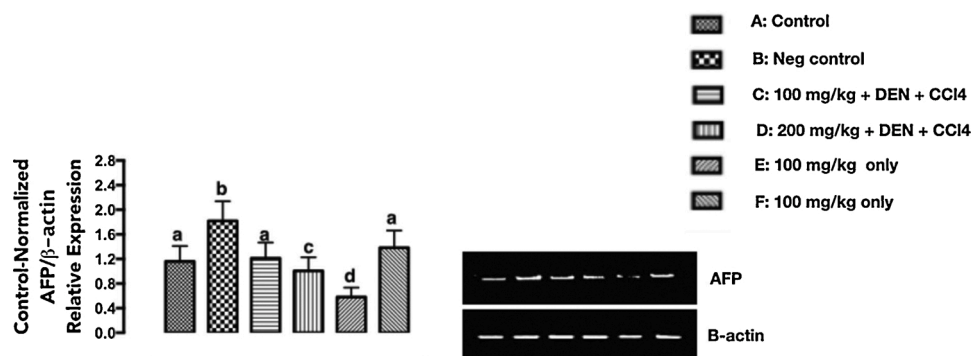
#### 3.4.5. Expression of Farnesyltransferase (FNTA) mRNA

In Fig. 8, the expression of FNTA mRNA was significantly up-regulated in the negative control group (B) as against group A. Its

expression was significantly downregulated in groups C, D, E, and F when compared with groups A and B.

#### 3.4.6. Expression of Alpha-fetoprotein (AFP) mRNA

In Fig. 9, the expression of AFP mRNA was up-regulated in group B. Meanwhile, the expression of AFP mRNA was significantly down-regulated in groups D and E as compared with both control groups (A and B). However, there was no significant expression of AFP mRNA in groups C and F when compared with group A. The expression of AFP



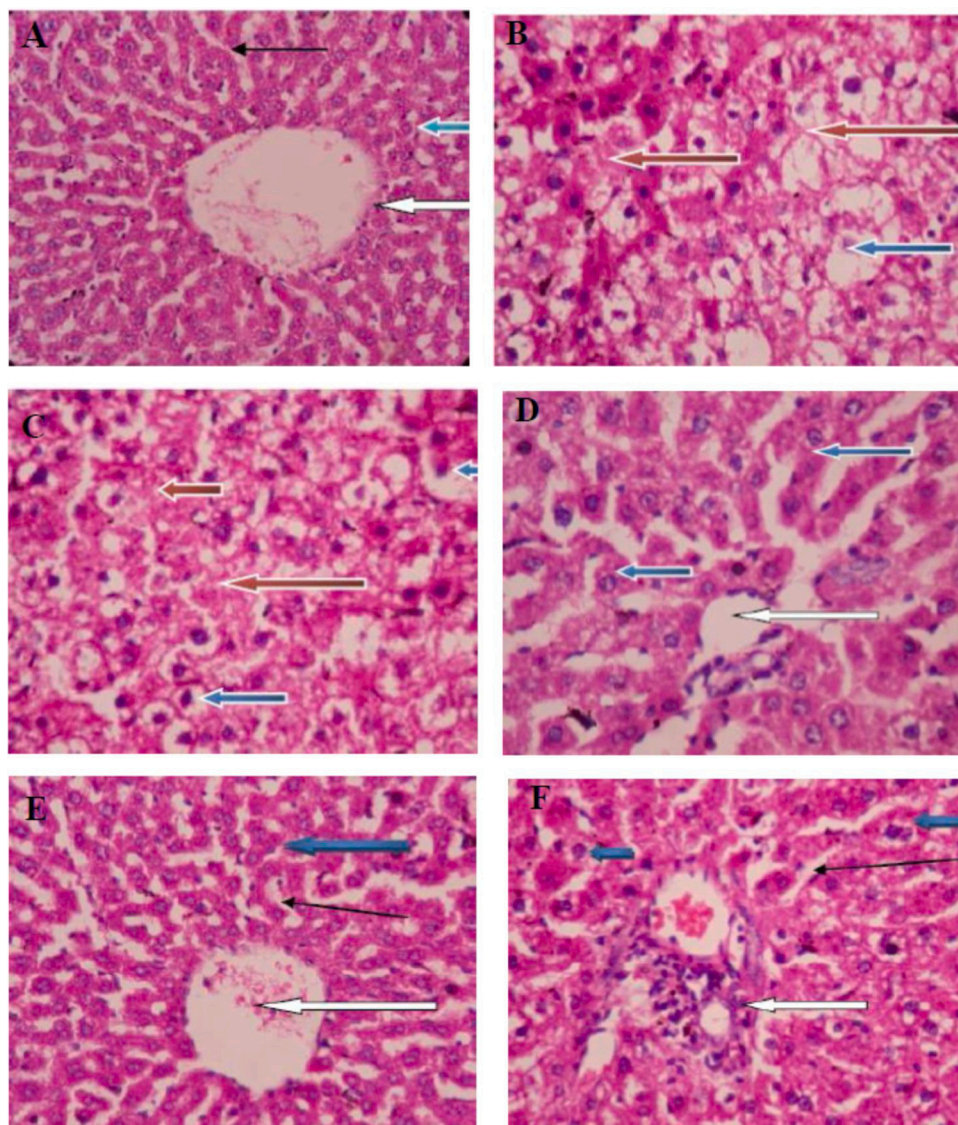
**Fig. 9.** Relative expressions of AFP mRNA in the liver of N-Diethylnitrosamine induced-hepatocellular carcinoma rats following treatment with *A. senegalensis* extract. Bars with different alphabet depict significant differences. Bars with the same alphabet show no significant difference.  $p < 0.05$  is significant.

mRNA in groups C, D, E, and F were significantly downregulated when compared with group B.

#### 4. Discussion

A total of sixty (60) phytochemicals were identified from the n-

hexane crude extract (see Table S1). Oleoyl chloride (9.7 %) constitute the highest percentage of phytoconstituents. The antimicrobial and endotoxin-neutralizing effects of Oleoyl compounds have been reported [49]. Also present in the N-hexane crude extract are; 912-Octadecadienoic acid (Z,Z)-, 2-hydroxy (7.89 %), performic acid (4.89 %), erythritol (4.47 %), 912-Octadecadienoic acid (Z,Z)- (4.30 %), 9-Octadecenoic



**Fig. 10.** Photomicrograph of liver tissues (H and E, x400) of rats A) with normal liver morphology in the control group, B) induced with the toxicant, C) treated with 100 mg/kg, D) treated with 200 mg/kg, E) administered 100 mg/kg, F) administered 200 mg/kg body weight of *A. senegalensis* extract. A) liver section of rat of the normal control showing normal liver morphology (blue arrow); the central venules appear normal (white arrows), the sinusoids appear normal (slender black arrow) without infiltration of inflammatory cells B) liver section showing cytoplasmic fat infiltration (blue arrow) and necrotic hepatocyte (red arrow). C) liver section showing normal venules (white arrow), sinusoids appear normal (slender arrow) with the absence of inflammatory cell infiltrations D) liver section with normal central venules (white arrow), the sinusoids show the focal area of mild inflammatory cells aggregate (slender arrow) with normal hepatocytes features (blue arrow) E) liver section showing normal central venules, sinusoids (white and thin arrows) F) liver section showing normal hepatocyte morphology and normal central venules (blue and white arrows respectively) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



acid (Z)-, 2-hydroxy-1-(hyd (4.16 %), 9-Octadecenoic acid (Z)-, methyl ester (3.62 %) (Table S1). The anticancer potentials of 9, 12-Octadecadienoic acid (Z, Z)-, 2-hydroxy, performic acid, and 9,12-Octadecadienoic acid are have all documented [50–52].

Diethylnitrosamines are acute carcinogens and hepatotoxins. It has been documented to arbitrate deleterious effects in tissues via induction of oxidative stress [53]. In our study, the anticancer potential of *A. senegalensis* (AS) leaf extract on DEN-induced hepatocellular carcinoma in rats was investigated. DEN caused a significant ( $p < 0.05$ ) increase in serum AST, ALT, ALP, and LDH levels when compared with the control group (Fig. 2). A rise in serum levels of these enzymes has been linked to detrimental liver architecture integrity as they leak into the bloodstream due to cellular damage [54]. Treatment with *A. senegalensis* decreased the enzyme levels significantly ( $p < 0.05$ ) when compared to DEN-induced untreated group, which suggests that AS could effectively impede DEN-induced liver cell damage. These results corroborate with the histological findings where the hepatocytes of the DEN-induced untreated group showed traces of cytoplasmic fat infiltration (blue arrow) and degeneration; and necrotic hepatocytes were also seen (red arrow; Fig. 10b) while the groups treated with AS showed liver section with normal venules (white arrow; Fig. 10c) with normal hepatocytes and liver morphology (blue arrow; Fig. 10d). However, group F (200 mg/kg b.wt of extract only) showed a significant increase in ALT, AST and LDH when compared with normal control (grp A), but there is no correlation between the biochemical result and histology results as the histology shows the liver tissues in group E and F with normal hepatocyte morphology and normal central venules.

Studies have shown that cancerous cells shift from aerobic to an anaerobic mechanism where glucose is converted to lactate. Since LDH catalyzes the conversion of pyruvate to lactate, it, therefore, contributes to tumor progression by renewing  $\text{NAD}^+$  supply for the glycolytic pathway, reducing pH for tumor invasion and thus activating immune response [55]. Langhammer et al. [56] and Brown et al. [57] described the expression of LDH in colon and breast tissue cancer. Lactate dehydrogenase A (LDHA), an isoform of LDH was found to be up-regulated in clinical samples of human hepatocellular carcinoma (HCC) [58]. Hence, this corroborates with the result observed in this study where LDH levels were significantly ( $p < 0.05$ ) elevated in DEN-induced untreated group (B), which could be needed for the tumor progression. AS significantly ( $p < 0.05$ ) reduced LDH levels in the treated groups, which suggests that LDH could be a promising target for averting and treatment of cancer (Fig. 2d); as LDH has been reported to be a key player and potential target in cancer therapy [59].

Oxidative stress entails the disparity between production and buildup of reactive oxygen species (ROS) in tissues and/or cells. When an imbalance occurs, there is an elevation of ROS production, and then lipid peroxidation prevails [60]. Increased MDA indicates the escalation of lipid peroxidation since it is a peculiar biomarker of oxidative stress [61]. Significant elevation of MDA levels observed in the untreated DEN-induced group (B), which could be a result of the overproduction of ROS and inadequate antioxidant defense; however, AS significantly reduced the MDA levels in a dose-dependent manner (Fig. 3a). The result of this study is in line with other studies where elevated lipid peroxidation was observed in tumors and malignant cells, including breast and liver tissues [62,63]. However, our findings were contradictory to that of Punnonen et al. [64] where they reported lower levels of thiobarbituric-acid reactive material in the cancerous tissue of breast cancer patients.

Superoxide dismutase (SOD) is a metalloenzyme that converts superoxide radicals to hydrogen peroxides. With high oxidative metabolism, it is spread out in the cells, protecting them from the harmful effect of superoxide anions [65]. In this study, decreased activity of SOD in DEN-induced untreated rats (grp B) was observed while the extract-treated groups showed significant ( $p < 0.05$ ) increase nearing normal levels (Fig. 3c); this corresponds to previous studies where diminished activity of SOD has been linked to cancer cell progression

[66,67]. Increased expression of SOD activity in the treated groups may be a way to recoup and protect the body from oxidative stress.

In contrast, the decreased activity suggests a higher consumption of antioxidative enzymes. GSH and GPx also protect against damage by providing a first-line defense and eliminating the reactive intermediates via conjugation [68,69]. As shown in Fig. 3, there was a decrease in the liver GSH (Fig. 3b) and GPx (Fig. 3d) in group B. The treated groups (C and D), however, showed a significant ( $p < 0.05$ ) rise in the GSH concentration only, although a minor (not notable) increase in GPx was also observed; suggesting the extract defense against free radicals.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that intervenes in infections, diseases, and cancer [70]. IL-6 in hepatocellular carcinoma (HCC) is documented to be a prognostic and distinct marker in cancer studies [71,72]. The up-regulation of IL-6 mRNA in group B (Fig. 4) corroborated the report of Li et al. [73] that the expression of IL-6 aids the progression of hepatocellular carcinoma. Hepatocellular carcinoma-inducing potential of DEN/ $\text{CCl}_4$  is hinged on pro-inflammation [74] hence the upregulation of IL-6 mRNA. The anti-inflammatory potentials demonstrated by *A. senegalensis* extract herein is in tandem with the report of Yeo et al. [30] that *A. senegalensis* possess anti-inflammatory potentials. Increased expressions of IL-6 in our study show HCC development can be explained with a simultaneous increase in the epidermal growth factor (EGF) gene expression in the negative control group. In a study designed to understand tumorigenic effects of EGFR signaling pathway in Kupfer cells, it was shown that DEN administration might have activated the expression of IL-6 which also link the high expression of IL-6 in HCC progression to increased expression of EGF as EGF deficiency failed to promote the expression of IL-6 [75]. This was also corroborated with the suggestion that Toll-like receptor and EGFR pathways are triggered during liver carcinogenesis, thus activating the expression of tumor necrosis factor (TNF) and interleukin-6 (IL-6) [76].

Apoptosis is an essential systematic process in which chronic liver damage can be regulated by BCL-2 proteins. While the pro-apoptotic genes instigate apoptosis, anti-apoptotic genes, including BCL-2, inhibit apoptosis [77]. X-linked Inhibitor-of-apoptosis Protein (XIAP) could play a crucial role in blocking the extrinsic and intrinsic pathways in apoptosis [78,79]. XIAP and BCL-2 are anti-apoptotic proteins and are overexpressed in malignancy [80]. The up-regulation of BCL-2 and XIAP mRNAs in group B (Fig. 5) demonstrated the resistance of malignant cells to apoptosis due to the co-administration of DEN and  $\text{CCl}_4$  which can be associated with increased gene copy number, transcription and translational processes of BCL-2 protein as seen in different cancer-related case [81,82]. It is worthy of note that treatments with the extract significantly downregulated the expressions of BCL-2 and XIAP mRNAs (Fig. 5). The proapoptotic tendencies demonstrated by *A. senegalensis* extract in the present study conforms with the earlier report by Adisa et al. [83] that *A. senegalensis* extract demonstrated apoptosis in HeLa cells.

The inverse expression of vascular endothelial growth factor (VEGF) compared to that of p53 in negative control (B) reported in our study could also be attributed to VEGF role in angiogenic pathway leading to HCC. VEGF has been suggested to enhance HCC through alteration in the PKC $\alpha$ /p53 pathway [84]. Activation of angiogenesis and reduced survival rate in the growth and progression of HCC has also been reported [85]. In addition, hypoxic conditions also resulted in increased VEGF protein concentration [86]. This hypoxia-induced VEGF increased expression has thus been described as an essential step in HCC related angiogenesis [87]. Thus, the increased VEGF expression observed in the DEN +  $\text{CCl}_4$  induced group (group B) could have been activated by an increased expression of HIF, which was also reported in the study (Fig. 6). *Annona senegalensis* extract demonstrated anti-angiogenic properties as observed in this study, EGFR, HIF-1, and VEGFR mRNAs were significantly downregulated in groups C and D in comparison with group B (Fig. 6). EGFR, HIF-1, and VEGF angiogenic related genes have all been implicated in pathogenesis and development of neoplasm [88].



The anti-angiogenic potentials demonstrated herein by the *Annona senegalensis* extract is in tandem with the report of Yi et al. [89] that *Annona atemoya*, a family (Annonaceae) of the *Annona* demonstrates anti-angiogenic potentials.

p21 induces cell cycle arrest, and it is overexpressed in the presence of oxidative stress [90,91]. The expression of p21 mRNA was significantly up-regulated in the group administered DEN/CCl<sub>4</sub> (group B) when compared with other groups (Fig. 7a); this may be due to oxidative stress associated with the toxicant (DEN/CCl<sub>4</sub>) [74]. It suffices to say that 100 mg/kg body weight of *A. senegalensis* (group C) significantly downregulated the expression of p21 mRNA; hence *A. senegalensis* extract demonstrates cell cycle arrest. The upregulation of the tumor suppressor p53 mRNA in group C as compared to group B depicts *A. senegalensis* might bring about its anticancer activities by up-regulating the p53 tumor suppressor mRNA (Fig. 7b).

The tumor suppressor gene (p53) plays a crucial role in tumor suppression through the regulation of the cell cycle. However, its protective role could be lost due to the co-administration of the carcinogens. This loss of function, which has been reported in various cancer studies, could be linked to “trans-dominant suppression” of the protein expression. Despite the ability of p53 to be oncogenic through mutation [92], the reverse was the case in our study. The “trans-dominant suppression” of p53, as reported, may have been a result of its interaction and formation of a heteromer with a mutant form of p53, thus causing a complete loss of function of the non-mutated p53 gene [93]. BCL-2 and p53 genes were part of the earliest discoveries in cancer research, thus giving a possible relationship between the two. The regulation of BCL-2 by p53 occurs through various mechanisms involving direct or indirect activation of BCL-2 genes. Despite different mechanisms involved, reduced expression of p53 coupled with an increased expression of BCL-2 could be linked to apoptosis regulation, where the low expression of p53 can be attributed as activation of liver tumorigenesis [94]. Despite a possible relationship between p53 and XIAP expression, as described by Li et al. [95], we observed differential expression between the two genes when compared to the normal group. The dual functional role of p53 explained earlier as either oncogenic or has its expression suppressed [93]. We cannot, however, confirm a non-existent relationship between the two genes. Our extract at 100 mg/kg (group E) effectively increased the expression of p53 (Fig. 7b).

Farnesyltransferases (FNTA) are post-translational modification proteins responsible for the oncogenic transformation of proteins. Inhibition of farnesyltransferase is shown to demonstrate anticancer properties [96]. Blocking Ras activation through inhibition of farnesyltransferase has been shown to induce growth arrest in neoplastic cells [97]. Interestingly, treatments with *A. senegalensis* extract significantly downregulate FNTA mRNA expression in comparison with both controls (A and B). Furthermore, the expression of FNTA mRNA in groups E and F were equally downregulated by *A. senegalensis* extract when compared with groups A and B (Fig. 8). The present results showed that *A. senegalensis* possess inhibitory roles on farnesyltransferase. The reported anticancer properties of *A. senegalensis* is likely due to its inhibition of farnesyltransferase activity [83].

Alpha-fetoprotein (AFP) is an established biomarker of hepatocellular carcinoma (HCC) [98]. The significant upregulation of AFP mRNA in group B is believed to be a result of tumor-induced by DEN and CCl<sub>4</sub>. The significant downregulation in the expression of AFP mRNA in all the treatment groups (C and D) and groups E and F by *A. senegalensis* extract further give credence to its anticancer potentials (Fig. 9) [83].

In conclusion, we demonstrated that the n-hexane extract of *A. senegalensis* has a protective ability against DEN-induced hepatocellular carcinoma, and this protection is accomplished by counteracting the induction of oxidative stress by DEN via increasing the activity of SOD, GSH and GPx and down-regulation of specific genes. Further research can be tailored towards mechanism and possible drug development from the extract of *A. senegalensis*.

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## Declaration of Competing Interest

The authors declare no conflict of interest in the article titled “*Annona senegalensis* extract demonstrates anticancer properties in N-diethylnitrosamine induced hepatocellular carcinoma in male wistar rats”.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.110786>.

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